DAIDS

VIROLOGY MANUAL

FOR HIV LABORATORIES

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Compiled by

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and

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HIV DRUG SUSCEPTIBILITY ASSAY

(With Addenda I and II)

I. PRINCIPLE

The *in vitro* drug susceptibility assay measures the extent that a drug inhibits HIV p24 antigen production by PBMC acutely infected with a viral isolate. It is performed in 96-well plates with a defined previously titrated inoculum of a clinical isolate to minimize inoculum effects. The infectivity of each clinical isolate is determined prior to drug susceptibility testing using a streamlined endpoint dilution assay that is analyzed by the Spearman-Karber statistical method. Both the infectivity titration and susceptibility determination use PHA-stimulated PBMC from normal donors.

After the infectivity of a virus stock is quantified, 1000 50% tissue culture infectious doses ($TCID_{50}$) per million PHA-stimulated PBMC is used as inoculum in a second set of *in vitro* infections. Infected wells (in the absence of drug and at each of a number of drug concentrations) are refed with a 50% medium exchange after 4 days of culture, and supernatant fluid is harvested after 7 days. HIV p24 antigen is quantified and the 50% inhibitory concentration (IC_{50}) of the drug is determined using the median effect equation.

II. SPECIMEN REQUIREMENTS

Cell-free supernatant is obtained from a positive HIV culture of patient PBMC, plasma, body fluid or tissue. This may originate from a qualitative macrococulture, a qualitative microculture or a quantitative microculture. This is referred to as an "isolate" or "viral stock". The stock from microcultures are likely to require some expansion to reach adequate levels of infectivity for the susceptibility assay. A standardized method for specimen collection/stock expansion for the drug susceptibility assay, using what are called "ministocks" has been developed (see "Specimen Processing" and "Specimen Codes"). In order to assess development of resistance to a therapeutic agent, it is optimal to test paired serial isolates in the same run. One should be from a sample collected prior to or at the beginning of therapy and the other a sample collected while on therapy or after therapy has been completed.

III. REAGENTS

All reagents are prepared using deionized water, reagent grade I.

Sterile Phosphate Buffered Saline (PBS) or sterile Hank's Balanced Salt Solution (HBSS) without calcium or magnesium. Store at room temperature. Note manufacturer's outdate or discard one week after opening.

Penicillin - available in $5x10^6$ unit vials. Store at room temperature. Observe manufacturer's outdate.

a. Add 25 mL of sterile water to the vial. Mix until contents are dissolved. Final concentration = 200,000 units/mL

b. Divide into 0.32 mL aliquots in sterile 1.5 mL microcentrifuge tubes and freeze at -20^{0}C in a labeled box. Label with a 1 year outdate, or manufacturer's outdate, whichever comes first.

Gentamicin - available in 50 mg/mL bottles. Open bottles under laminar flow hood only; divide into 0.640 mL aliquots in sterile 1.5 mL microcentrifuge tubes. Store unopened bottles at room temperature; store aliquots at 4^{0}C. Outdates one month after opening.

Fetal Bovine Serum (FBS) - available in 500 mL sterile bottles from various manufacturers. Store frozen at -20^{0}C. Note manufacturer's outdate. When needed, rapidly thaw a bottle in a 37^{0}C water bath, then heat-inactivate in a 56^{0}C water bath for 30 minutes with occasional shaking. The level of H_2O in the water bath should be as high as the level of the serum in the bottle. Store at 4^{0}C after thawing. Heat-inactivated FBS has a one month outdate.

RPMI 1640 medium with L-glutamine (2 mM) - Store at 4^{0}C and observe manufacturer's outdate.

IL-2 (interleukin-2) - available in 50 mL bottles from Boehringer Mannheim at a discounted price for labs within the ACTG. Store at -20^{0}C. Note manufacturer's outdate. As needed, thaw a 50 mL bottle (freeze the remaining 25 mL).

Basic Medium:

To make 620 mL:

a. Add 120 mL heat-inactivated FBS to 500 mL of RPMI 1640 medium with L-glutamine. Final concentration (120/620) is approximately 20%.

b. Add 310 µL stock penicillin. (Concentration of penicillin used is $5x10^6$ units/25 mL or 200,000 units/mL; 0.31 mL of 200,000 units/mL = 62,000 units and 62,000 units/620 mL final volume of medium=100 units/mL for final concentration).

c. Add 620 µL Gentamicin. (Concentration of Gentamicin used is 50 mg/mL or 50 µg/µL; 620 µL of 50 µg/µL = 31,000 µg and 31,000 µg/620 mL final volume of medium = 50 µg/mL for final concentration).

Store Basic Medium at 4^{0}C for up to 1 month.

Growth Medium (culture medium) - also called IL-2 Medium or T-Cell Growth Factor (TCGF) Medium.

To make 500 mL:

a. 475 mL Basic Medium.

b. 25 mL IL-2. (Final concentration = 25 mL/500 mL = 5%.)

Store Growth Medium at 4^0C for up to 1 month. Growth Medium should warmed before use.

Trypan Blue Stain - this stains non-viable cells dark blue, and is used to determine the viable cell count of a culture. Prepare a 0.4% solution by adding 0.4 gm Trypan Blue (available from Sigma) and 1 mL Glacial Acetic Acid to 99 mLs distilled H_20 or saline. After dissolving, filter solution through Whatman filter paper or a 0.45 µ filter.

PHA-stimulated uninfected donor PBMCs - see procedure for Preparation of PHA-stimulated, Uninfected Donor Peripheral Blood Mononuclear Cells (PBMC).

Antiretroviral Drug Concentrations. Drug should be stored at -20^0C to -85^0C depending on drug (e.g., protease inhibitors should be stored at -85^0C) in borosilicate glass vials. Avoid storing drug at low concentrations.

Drug-sensitive and drug-resistant isolates can be obtained from the NIH AIDS Research and Reference Reagent Program.

IV. SUPPLIES AND EQUIPMENT

Gloves
Disposable lab coat
Laminar flow hood (Class 2 biosafety hood)
Sterile 2, 5 and 10 mL pipette
Hemacytometer
96-well, flat bottomed tissue culture plates
Sterile 1.5 and 0.5 mL microcentrifuge tubes
20 µL, 200 µL, and 1000 µL micropipettor
Sterile 50 µL, 200 µL and 1000 µL pipette tip
Multichannel 50 µL, 200 µL micropipettors
Repeat pipettor and sterile tips
Borosilicate glass tubes
1% bleach or suitable disinfectant
Low speed centrifuge with O ring sealed safety cups
Compound microscope
CO_2 incubator (37 ± 1^0C with humidity)
37^0C and 56^0C water baths

V. PROCEDURE

A. Virus Stocks: Collect cell-free, virus-containing supernatants from positive HIV cocultures (patient, drug-sensitive, and drug-resistant control viral isolates) according to standard methods found in others sections of the Manual (See "Specimen Processing" and "Specimen Codes").

NOTE: SUBSEQUENT PROCEDURE SHOULD BE PERFORMED IN A CLASS 2 BIOSAFETY LAMINAR FLOW HOOD USING STERILE TECHNIQUE AND ADHERING TO CDC/NIH STANDARDS (INCLUDING USE OF GLOVES AND LAB COATS).

B. Virus Stock Infectivity Titration: Seven serial four-fold dilutions of virus stock, ranging from 1:16 through 1:65,635, are titrated in triplicate in 96 well flat-bottomed tissue culture plates.

1. Centrifuge 1 to 3 day old PHA-stimulated donor PBMC at 400 x g for 10 minutes at 20^0C to 24^0C, remove and discard supernatant, then resuspend cells in Growth Medium and enumerate cells. Determine viability with 0.4% trypan blue exclusion dye; do not use cells if viability is less than 85%. Adjust sample with Growth Medium to a concentration of $4x10^6$ cells/mL (exactly 4.2 million cells are needed per plate as currently formatted; adjust volume accordingly). Keep in CO_2 incubator at 37^0C until step 6.

2. Add 200 µL PBS or HBSS to all wells labeled P, see "Plate Format" below.

PLATE FORMAT FOR HIV TITRATION

	1	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**	**11**	**12**
A	P	P	P	P	P	P	P	P	P	P	P	P
B	P	P	P	P	P	P	P	P	P	P	P	P
C	P	P	4^{-2}	4^{-3}	4^{-4}	4^{-5}	4^{-6}	4^{-7}	4^{-8}	P	P	P
D	P	P	4^{-2}	4^{-3}	4^{-4}	4^{-5}	4^{-6}	4^{-7}	4^{-8}	P	P	P
E	P	P	4^{-2}	4^{-3}	4^{-4}	4^{-5}	4^{-6}	4^{-7}	4^{-8}	P	P	P
F	P	P	P	P	P	P	P	P	P	P	P	P
G	P	P	P	P	P	P	P	P	P	P	P	P
H	P	P	P	P	P	P	P	P	P	P	P	P

3. Add 150 µL Growth Medium to the wells labeled 4^{-3} to 4^{-8} (rows C to E, columns 4 to 9) with a multi-channel micropipettor.

4. Rapidly thaw an aliquot of the virus stock at 37^0C in a water bath until only a small crystal of ice remains. Immediately dilute the sample 1:12 in Growth Medium (e.g., 0.1 mL of virus stock to 1.1 mL of culture medium) and transfer 200 µL to each well labeled 4^{-2} (column 3 in rows C to E).

5. With a multi-channel pipette, transfer 50 µL from wells labeled 4^{-2} to wells labeled 4^{-3} (column 3 to column 4 in rows C to F). Continue such transfers, moving from *left* to *right*, changing tips prior to mixing contents of the next column of wells. Discard 50 µL from the wells labeled 4^{-8} (column 9).

6. Dispense 50 µL of PBMC (200,000 cells) from step 1 to all wells containing viral stock, moving from *right to left.*

7. Cover the plate and incubate at 37^0C, 5% CO_2 with humidity.

8. On day 4, with a multi-channel pipette, moving from *right to left* across the plate, resuspend the cells in each culture well by mixing and remove and discard 125 µL of the cell suspension. Add 150 µL of fresh Growth Medium back to each well, again moving from *right to left* . Return the plate to the incubator.

9. On day 7, the HIV titration assay is terminated and the appropriate supernatants are tested for HIV p24 antigen as follows:

    a. Transfer 100 µL of supernatant from the titration wells to wells of a HIV p24 antigen plate that contains 100 µL of Growth or Basic Medium and 20 µL of the manufacturer's disruption buffer containing Triton X-100.

    b. The assay is performed according to the recommendations of the manufacturer using the VQA reference standard and QC check samples as described in "Standard HIV p24 Antigen Assay". A well is scored "positive" if the VQA corrected value is 50 pg/mL.

10. The $TCID_{50}$ is calculated by the Spearman-Karber method. An example of this calculation follows:

a. Scoring the HIV p24 antigen plate

	1	**2**	**4^{-2}**	**4^{-3}**	**4^{-4}**	**4^{-5}**	**4^{-6}**	**4^{-7}**	**4^{-8}**	**10**	**11**	**12**
A	P	P	P	P	P	P	P	P	P	P	P	P
B	P	P	P	P	P	P	P	P	P	P	P	P
C	P	P	+	+	+	+	-	-	-	P	P	P
D	P	P	+	+	-	-	-	-	-	P	P	P
E	P	P	+	+	+	-	-	-	-	P	P	P
F	P	P	P	P	P	P	P	P	P	P	P	P
G	P	P	P	P	P	P	P	P	P	P	P	P
H	P	P	P	P	P	P	P	P	P	P	P	P
	r = 0	0	1	2	3	3	3	= 12				

b. Calculating $TCID_{50}$/mL

xk = dose of highest dilution
r = number of "-" responses
d = spacing between dilutions
n = wells per dilution
r = sum of r

Spearman-Karber formula: $M = xk + d [0.5 - (1/n) (r)]$
$= 8 + 1 [0.5 - (1/3)(12)]$
$= 8 + 1 (0.5 - 4.0)$

The 50% endpoint is $4^{-4.5}$
Converting to 10^x:
$x = 4.5*\log 4$
$= 4.5* 0.602$
$= 2.7$ the 50% titer is $10^{2.7}$

To calculate the $TCID_{50}$/mL of virus stock, the original dilution must be corrected by multiplying by 5 (1000 µL÷ 200 µL):

$TCID_{50}/mL = 5* 10^{2.7}$
$= 10^{0.70} * 10^{2.7}$
$= 10^{3.4}$
$TCID_{50}/mL$ - 2.51×10^3

C. Virus Susceptibility Testing Assay (for ZDV)

1. Prepare a 2X working solution by diluting the drug of interest in Growth Medium. (For ZDV, a 1 mM stock solution is diluted to yield concentrations of 10.0 µM, 2.0 µM, 0.2 µM, 0.02 µM and 0.002 µM in at least 1 mL of Growth Medium).

2. With a multi-channel pipette, add 200 µL PBS to all wells labeled P, (see Plate Format below).

3. With a multi-channel pipettor, add 100 µL Growth Medium to wells labeled "0" (C to E in column 3). Add 100 µL of 2X ZDV working solutions to their respective wells labeled with the 1X drug concentration (columns 4 to 8). The numbers in the illustration are the final concentrations of ZDV in µM. The remaining working solutions may be stored at -20^{0}C until day 4 or made up fresh at the time. Peptides, such as protease inhibitors, should be prepared fresh each time. (See Plate Format below.)

PLATE FORMAT FOR DRUG SUSCEPTIBILITY TESTING

	1	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**	**11**	**12**
A	P	P	P	P	P	P	P	P	P	P	P	P
B	P	P	P	P	P	P	P	P	P	P	P	P
C	P	P	0	.001	.01	.1	1.0	5.0	P	P	P	P
D	P	P	0	.001	.01	.1	1.0	5.0	P	P	P	P
E	P	P	0	.001	.01	.1	1.0	5.0	P	P	P	P
F	P	P	P	P	P	P	P	P	P	P	P	P
G	P	P	P	P	P	P	P	P	P	P	P	P
H	P	P	P	P	P	P	P	P	P	P	P	P

4. Sediment 1 to 3 day old PHA-stimulated normal donor PBMC at 400 x g for 10 minutes at 20 to 24^{0}C, remove and discard supernatant, then resuspend cells in Growth Medium and enumerate cells. Adjust the sample with Growth Medium to a concentration of 4 million PBMC/mL. The viability should be determined by trypan blue exclusion and should not be used if the viability is < 85%.

5. Add 1.0 mL of these donor cells to a sterile 15 mL conical centrifuge tube and centrifuge the cells at 400 x g for 10 minutes at 20 to 24^{0}C. Remove the supernatant and add the required amount of titrated virus stock (1000 $TCID_{50}/10^6$ PBMC, therefore 4000 $TCID_{50}/4x10^6$ PBMC). The final volume should be kept to 1 mL; hence the minimum useable titer is 4000 $TCID_{50}$/mL. After mixing gently, the suspension is incubated for 1-3 hours at 37^{0}C, 5% CO_2 with humidity. Longer incubation times are recommended for lower titer virus stocks. Example Calculation:

$\frac{4000 \text{ (desired TCID}_{50}\text{/mL)}}{8000 \text{ (actual TCID}_{50}\text{/mL of virus stock)}} = 0.5$ mL (vol. of virus stock needed)

6. After incubation, bring volume up to 2 mL for $4x10^6$ cells and dispense 100 TL of the infected cells into each well of columns 3 to 8 in rows C to E. Each well will have 0.2 X 10^6 PBMC.

7. The final volume in each well should be 200 µL. Incubate the plates at 37^0C, 5% CO_2 with humidity.

8. On day 4, examine the cells microscopically for obvious cytopathic effect (CPE), then thaw the 2X working solutions (or prepare fresh material). Add 0.5 mL of the 2X working solutions to 0.5 mL of Growth Medium to give 1X working solutions. Moving from *left to right* across the plate, mix, remove and discard 125 µL of cell suspension from the culture wells. Then add 150 µL of each of the 1X ZDV concentration (or other antiretroviral agent) working solutions to the appropriate wells (see plate format).

9. On day 7, prepare dilutions of the wells for HIV p24 antigen evaluation as follows:

    a. With a multi-channel pipettor, add 205 TL of Growth Medium, followed by 25 µL of the manufacturer's disruption buffer containing Triton X-100 to the wells in columns 1 to 6 of rows C through H of a <u>new</u> 96-well, flat bottomed polystyrene plate. This will be the Dilution Plate and the 36 wells just pipetted will accommodate a 1:12.5 and 1:156 dilution of the Drug Susceptibility Plate.

    b. Transfer 20 µL of supernatant from each of the drug susceptibility assay wells (rows C, D, and E of the Drug Susceptibility Plate) to their respective rows in the Dilution Plate (rows C, D, and E), using a multi-channel micropipettor (1:12.5 dilution). Mix and then transfer 20 µL from row C to row F, row D to row G, and row E to row H (total dilution of 1:156). Cover the plate with a 5 x 8 inch low-density polyethylene bag or similar product to prevent drying of the samples. Store the plate at -30^0C or lower and run the HIV p24 antigen assay within 72 hours.

10. Determine the HIV p24 antigen concentration of the diluted wells as follows:

    a. Thaw the plate with the diluted samples at ambient temperature. Transfer 20 µL from each well in rows F to H to the appropriate well or tube of the HIV p24 antigen assay kit containing 180 µL of Growth Media (final

dilution of 1:1560). A range of dilutions may need to be tested until the untreated control (0 µM drug concentration) optical densities lie on the linear slope of the calibration curve. (For example, a final dilution of 1:1000 may be better, in general, for some labs.)

b. Follow the manufacturer's procedure for HIV p24 antigen detection.

c. An extended VQA calibration curve that ranges from 400 pg/mL to 25 pg/mL in two-fold dilutions should be run. Results will be analyzed using a quadratic curve fit for the Coulter and Dupont p24 antigen assays. Analysis for the Abbott kit is not modified.

11. Calculate 50% inhibitory concentration (IC_{50}) by the median effect equation and report both raw and analyzed data as follows:

a. The median effect equation of Chou and coworkers, can be used in one of the following ways:

1) the exponential form of the equation can be used and a curve fit to the data points (F_a and drug concentration) using nonlinear regression:

Fraction affected: $(F_a) = 1/[1 + (IC_{50}/\text{drug concentration})^m]$

m = slope of the curve

Fraction affected: (F_a) = % reduction from untreated control x 0.01

Systat (Systat, Inc., Evanston, IL) can solve for IC_{50} and m simultaneously using nonlinear regression modeling. After the equation's constants (IC_{50} and m) are determined, a curve of F_a versus drug concentration can be constructed on a log-linear plot with drug concentration on the log scale of the x-axis.

2) logarithmic form of the equation can be used and a curve fit to the data points by linear regression:

$\text{Log } (F_a/F_u) = m \log [\text{drug concentration}] - m \log [IC_{50}]$

Fraction affected: (F_a) = % reduction from untreated control x 0.01
Fraction unaffected: (F_u) = % maximum x 0.01

b. "Dose effect analysis with microcomputers" by Chou and Chou (Biosoft, Ferguson, MO) calculates the IC_{50} using linear regression to solve for the best fit of the data to this logarithmic form of the median effect equation. When actual values of F_a/F_u and drug concentration are plotted on log-log

scales, the IC_{50} is the drug concentration that corresponds to y=1. The Chou and Chou software plots log (F_a/F_u) versus log (drug concentration) on linear scales, and the IC_{50} is the y intercept (since log 1 = 0). However, when using this method, F_u cannot be zero and therefore must be changed to a consistent arbitrary number if no drug effect is seen at low drug concentrations.

c. Other software can be used: (a) for nonlinear regression modeling of the exponential form of the equation, (b) for linear regression modeling of the logarithmic form of the equation, or (c) for plotting F_a versus drug concentration (or F_u versus drug concentration) after IC_{50} has been determined by solving the median effect equation.

VI. QUALITY CONTROL

A. Critical evaluation of HIV p24 antigen data derived from drug susceptibility testing is imperative.

1. A repeat infectivity titration and drug susceptibility assay should be performed if data suggest that levels of virus replication at day 7 in the virus susceptibility assay are unexpectedly either too low or too high. Each investigator will need to gain experience in recognizing these parameters that suggest extremely low or high levels of replication at day 7 in the virus susceptibility assay. Possible parameters are, as follows:

a. Suboptimal virus replication could be a possibility if an assay resulted in HIV p24 antigen levels $\leq$ 25 ng/mL in the untreated control wells. While such data may be analyzable to obtain an IC_{50}, it may not yield an IC_{50} comparable to that obtained from an assay that results in higher levels of p24 antigen.

b. The possibility that virus replication may have occurred at too high a level is suggested if evidence of early ballooning CPE is detected in drug-containing wells on day 4. In that case, the possibility should be considered that the day 7 harvest occurred after virus replication had peaked and that "breakthrough" of drug sensitive virus may have occurred in the presence of drug. For this reason, the protocol includes a microscopic examination of cultures for CPE on day 4.

B. The use of controls for donor PBMC variability in drug susceptibility testing is recommended.

1. A potential source of inconsistency in the drug susceptibility assay is the variability that is sometimes seen in the sensitivity of PBMC from different

donors to support the growth of HIV. When feasible, it is advisable to use a designated pool of donors in whose cells HIV-1 is known to replicate well. The optimum is to use the same donor's cells for infectivity titration and susceptibility testing of a particular isolate. The following optional controls can help to assess the influence of donor PBMC variability on the replicative activity of HIV-1 and the eventual calculation of IC_{50}.

a. With each batch of PBMC, inoculate 200 $TCID_{50}$ of a standard, well-characterized ZDV-resistant isolate (e.g., A018C) in triplicate wells containing 0, 0.1, and 1.0 µM ZDV. Expression of HIV-1 p24 antigen should be similar in all these wells for such a high-level ZDV-resistant isolate. Significant drug inhibition (>70% reduction at 1.0M relative to no drug, for example) raises the possibility of inadequate virus replication ("apparent" drug susceptibility) and calls into question the drug susceptibility testing using this batch of PBMC and/or the infectivity titration of the isolate.

VII. LIMITATIONS OF THE ASSAY

This assay does not discriminate reliably between ddI-susceptible and ddI-resistant isolates, or other agents not associated with at least 5 to 10 fold shifts in phenotypic drug susceptibility (e.g.,, d4T, ddC).

VIII. REFERENCES

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ADDENDUM I - HIV DRUG SUSCEPTIBILITY ASSAY WITHOUT TITRATION OF VIRAL ISOLATE

I. PRINCIPLE

ZDV drug susceptibilities of 47 viral isolates were determined in 7 laboratories using the standard method described above (HIV Drug Susceptibility Assay) and two modified methods which eliminate the titration step. In place of 1000 $TCID_{50}$ (determined by the titration step), 80-100 µL or 400-500 µL of viral stock supernatant were used to infect the donor PBMC in the Virus Susceptibility Assay. The assays were then performed as usual. When the IC_{50} from the standard and modified assays were compared, the majority of viruses were consistently classified as susceptible, intermediate or resistant by all three methods. The zidovudine IC_{50} values tended to be higher with the 400-500 µL input, therefore the lower input of 80-100 µL was chosen as a substitute for the standard 1000 $TCID_{50}$. This modification results in a faster and less expensive assay.

II. PROCEDURE

1. Omit section V.B Virus Stock Infectivity Titration from the standard procedure.

2. Begin with section V.C Virus Susceptibility Testing Assay (for ZDV). In step 5, substitute 100 µL of virus stock supernatant for the 1000 $TCID_{50}$ value that would have been added to the 4 X 10^6 donor PBMC. Continue with the standard assay as described.

III. REFERENCES

Erice A, Brambilla D, Demeter L, Penas JG, Brewster F, Stellrecht K, Reichelderfer PS, Mayers DL, Balfour HH, Japour AS, and the AIDS Clinical Trials Group Virology Committee Resistance Working Group. Simplified Susceptibility Assay for Human Immunodeficiency Virus Type 1 Clinical Isolates. Submitted.

ADDENDUM II - SIMPLIFIED HIV DRUG SUSCEPTIBILITY ASSAY

I. PRINCIPLE

Zidovudine susceptibilities for 525 clinical HIV-1 isolates were assessed using the standardized drug susceptibility assay. Zidovudine IC_{50} values were calculated both before and after reducing the number of replicates and zidovudine concentration testing in vitro. Excellent results were achieved when the 0.001 µM zidovudine concentration was omitted and when duplicate rather than triplicate wells were used at the remaining concentrations. In view of the high cost of this labor-intensive assay, it is concluded that such alterations of the existing protocol are valid and cost-effective.

II. PROCEDURE

A. In section V. C. 3. and related steps, set up the virus/drug concentration wells in duplicate instead of triplicate.

B. In section V. C. 1. and related steps, prepare and use only five concentrations of zidovudine (10.0 µM, 2.0 µM, 0.2 µM, 0.02 µM and 0 µM).

III. REFERENCES

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